

# Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice

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**Demas, Gregory E., Vladimir Chefer, Mark I. Talan, and Randy J. Nelson.** Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. *Am. J. Physiol.* 273 (*Regulatory Integrative Comp. Physiol.* 42): R1631–R1637, 1997.—Animals must balance their energy budget despite seasonal changes in both energy availability and physiological expenditures. Immunity, in addition to growth, thermoregulation, and cellular maintenance, requires substantial energy to maintain function, although few studies have directly tested the energetic cost of immunity. The present study assessed the metabolic costs of an antibody response. Adult and aged male C57BL/6J mice were implanted with either empty Silastic capsules or capsules filled with melatonin and injected with either saline or keyhole limpet hemocyanin (KLH). O<sub>2</sub> consumption was monitored periodically throughout antibody production using indirect calorimetry. KLH-injected mice mounted significant immunoglobulin G (IgG) responses and consumed more O<sub>2</sub> compared with animals injected with saline. Melatonin treatment increased O<sub>2</sub> consumption in mice injected with saline but suppressed the increased metabolic rate associated with an immune response in KLH-injected animals. Melatonin had no effect on immune response to KLH. Adult and aged mice did not differ in antibody response or metabolic activity. Aged mice appear unable to maintain sufficient heat production despite comparable O<sub>2</sub> production to adult mice. These results suggest that mounting an immune response requires significant energy and therefore requires using resources that could otherwise be allocated to other physiological processes. Energetic trade-offs are likely when energy demands are high (e.g., during winter, pregnancy, or lactation). Melatonin appears to play an adaptive role in coordinating reproductive, immunologic, and energetic processes.

energetics; melatonin; thermoregulation; immunoglobulin

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ANIMALS HAVE EVOLVED to maintain a balanced energy budget (7, 34). Balancing an energy budget becomes challenging during winter when food supplies dwindle yet thermoregulatory demands increase. Nontropical animals have evolved specific adaptations to cope with winter energy shortages. These adaptations serve to partition available energy to bodily functions where it is most needed (e.g., thermoregulation, cellular processes) (33). Thus during winter most available energy is partitioned into thermoregulation and not into growth, reproduction, or other nonessential processes.

In addition to these well-known energetically demanding biological processes, the immune system also requires energy to maintain its function. Mounting an antibody response likely requires using resources that could otherwise be allocated to other biological functions (1, 28). Thus immune function, like other biologi-

cal processes, should be “optimized” so that individuals can tolerate small infections if the energetic costs of mounting an immune response outweigh the benefits (3). Recent research on bighorn sheep has provided evidence for an energetic trade-off between the costs of immune and reproductive function (13). Lactation requires substantial energy. Lactating bighorn ewes demonstrate increased parasitic infection in fecal samples compared with nonlactating females (13). Increased parasitic infection is likely due to reduced immune function in lactating ewes, but this hypothesis remains to be tested directly. Thus optimal resource allocation between reproductive and immune function depends on competing energetic demands and their associated costs and benefits (28). Similar trade-offs presumably exist between immune function and other energetically demanding biological functions (e.g., thermoregulation and reproduction) (1, 10, 28).

Although energy availability can affect immune responses (e.g., Ref. 16), few studies have directly assessed the metabolic cost of mounting an antibody response. Presumably, the initiation of an immune response (i.e., inflammation, activation of cytokines, induction of fever) requires substantial energy, but this proposition has only been tested indirectly. For example, European kestrels (*Falco tinnunculus*) infected with the blood protozoan *Trypanosoma* increase their daily energy expenditure relative to uninfected birds (1). Chickens injected with sheep red blood cells (SRBC) consume more food but gain less weight than control chickens injected with saline (16). The increase in energy intake likely reflects increased energetic demands necessary for mounting an anti-SRBC antibody response. Depending on the species, every 1°C increase in body temperature requires a 7–13% increase in caloric energy production (18). The goal of the present experiment was to quantify the energetic costs of an immune response. The effects of aging on both metabolic activity and immune function were also assessed in the present study.

The aging process is associated with a progressive decline in a wide range of physiological and biochemical functions including both metabolic energy expenditure and immune function (26). The pineal hormone melatonin, secreted in a rhythmic pattern with elevated plasma levels occurring at night and basal levels during the day, has been implicated in the aging process (2, 23). For example, the nocturnal peak in plasma melatonin decreases dramatically with age (i.e., 40–60%) in rodents (29). The age-related decline in melatonin secretion may alter hypothalamic sensitiv-

ity to internal regulatory mechanisms (e.g., homeostatic processes), leading to internal desynchronization and thus accelerating the aging process (23). Consistent with this hypothesis, although controversial (e.g., Ref. 31), exogenous melatonin treatment reinstates internal synchrony in aged animals (2) and has been reported to prolong life span in experimental animals (22). Melatonin also affects immune function (16, 24). Melatonin generally enhances immune function; exogenous melatonin treatment enhances both humoral and cell-mediated immunity (reviewed in Refs. 15, 19). Thus age-related reductions in immune function may be correlated with changes in the pattern or amount of melatonin secretion (e.g., Ref. 23). In the present study, the energetic costs of an immune response as well as the effects of melatonin on both immune function and metabolic activity were assessed in both adult and aged mice. It was anticipated that mice immunized with keyhole limpet hemocyanin (KLH) would expend more  $O_2$  than saline-treated control mice. If melatonin enhances immune function, then melatonin-treated animals should demonstrate higher immunoglobulin G (IgG) responses compared with control animals. Also, it was predicted that aged mice would display weaker immune responses compared with adult mice.

#### MATERIALS AND METHODS

**Animals.** Eighty adult (10–12 mo of age) and 80 aged (22–24 mo of age) C57BL/6J mice were obtained from the animal colony maintained at the Gerontology Research Center. All animals were group housed (4/cage) in polypropylene cages ( $27.8 \times 7.5 \times 13.0$  cm) with 2–3 cm of wood shavings for bedding. The colony room was maintained with a 12:12-h light-dark cycle [lights on 0600 Eastern Standard Time (EST)]. Temperature was kept constant at  $22.5 \pm 1^\circ\text{C}$ . Food (NIH-07 formula, 24% protein, 4.2 kcal/g, in stainless steel hoppers) and tap water (from an automated filtering system) were available ad libitum throughout the experiment. The colony room was free from mouse viral infection throughout the experiment.

**Experimental methods.** Both adult and aged animals were divided into two experimental groups. The first group was implanted with a 15-mm-long Silastic capsule (1.47 mm ID, 1.95 mm OD, Silicone Medical Grade Tubing, American Scientific Product, McGraw Park, IL) filled 10 mm with melatonin crystals (Sigma, St. Louis, MO) and sealed with 2.5 mm of Silastic adhesive on each end. The amount of melatonin released from these capsules is relatively constant; about 5  $\mu\text{g}$  of melatonin is released per day (38). A control group was implanted with 15-mm empty Silastic capsules. Surgery was performed under light anesthesia with methoxyflurane vapors (Metofane, Pitman-Moore, Mundelein, IL). A 70% alcohol solution was applied to the intrascapular surface, and a 5-mm incision was made perpendicular to midline. Capsules were implanted and the incision was closed with a 9-mm autoclip (Clay Adams, Parsippany, NJ). Nitrofurazone antibacterial ointment (Phoenix Pharmaceutical, St. Joseph, MI) was applied to the skin surface to prevent infection. Animals were then returned to the colony rooms for a 1-wk recovery period.

Eight adult mice (4 melatonin treated and 4 control) and eight aged mice (4 melatonin treated and 4 control) were randomly selected from the experimental groups. The mice were weighed and colonic temperature was measured by a prelubricated thermoprobe (Physitemp IT-14) inserted 2.0–

2.5 cm into the rectum. Then animals were removed from the restraint tubes and placed directly into individual metabolic chambers (Oxymax Fast Response Chamber, 16 cm long, 5 cm ID) that were air-tight with the exception of two air valves. Fresh air was pumped into the chamber at a flow rate of  $\sim 1,600$  ml/min and the outgoing air was sent to  $O_2$  and  $CO_2$  sensors (Oxymax System, Columbus Instruments, Columbus OH). Once every 4 min,  $O_2$  consumption and  $CO_2$  production were measured, and the output was sent to a computer. Only  $O_2$  consumption data are reported in this paper. Metabolic activity was measured for 90 min (at 1000 EST) at room temperature ( $22.5^\circ\text{C}$ ) for 3 consecutive days. This provided a baseline assessment of metabolic function.

After initial baseline assessment, melatonin-treated and control animals were further divided into two groups. Experimental animals received a single subcutaneous injection of 150  $\mu\text{g}$  of the novel antigen KLH suspended in 0.1 ml sterile saline while control animals received injections of the saline vehicle alone. KLH is an innocuous respiratory protein derived from the giant keyhole limpet (*Meagthura crenulata*). KLH was used because it generates a robust antigenic response in rodents, but does not cause any adverse reactions (e.g., inflammation or fever) and does not make the animals ill (12). At 5, 10, and 15 days postimmunization, six animals from each group were randomly selected to have their metabolic activity monitored. Different animals were used for each of the three time points (e.g., 5, 10, and 15 days post-KLH injection). These days were chosen to capture peak immunoglobulin production during the course of the immune response (12). Body mass and colonic temperature were determined and their metabolic activity was monitored for 90 min using the Oxymax system as described above. Animals were given light anesthesia and a blood sample (500  $\mu\text{l}$ ) was obtained from the retroorbital sinus. Samples were allowed to clot for 1 h, the clot was removed, and samples were centrifuged (at  $8^\circ\text{C}$ ) for 1 h at 2,500 revolutions/min. Serum aliquots were extracted and stored in polypropylene microcentrifuge tubes at  $-80^\circ\text{C}$  until assayed. After blood sampling, all animals were killed by cervical dislocation. Paired testes, spleens, and brown adipose tissue (BAT) were removed, cleaned of connective tissue, and weighed.

**Enzyme-linked immunosorbent assay for IgG.** Serum concentrations of anti-KLH IgG were determined using an enzyme-linked immunosorbent assay. Microtiter plates were coated with antigen by overnight incubation at  $4^\circ\text{C}$  with 0.5 mg/ml KLH in sodium bicarbonate buffer, washed with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), blocked with 0.5% nonfat dry milk in PBS-T overnight at  $4^\circ\text{C}$ , and washed again with PBS-T. Thawed serum samples from mice were diluted 1:100, 1:200, 1:400, and 1:800 with PBS-T, and 150  $\mu\text{l}$  of each serum dilution was added in duplicate to the wells of the antigen-coated plates. Positive control samples (pooled serum from mice previously determined to have high levels of anti-KLH antibodies, similarly diluted with PBS-T) and negative control samples (pooled serum from mice never immunized with KLH) were also added in duplicate to each plate. The plates were sealed, incubated at  $37^\circ\text{C}$  for 3 h, and then washed with PBS-T. Secondary antibody (alkaline phosphatase-conjugated anti-mouse IgG diluted 1:2,000; Cappel, Durham, NC) was added to the wells, and plates were sealed and incubated at  $37^\circ\text{C}$  for 1 h. Plates were again washed with PBS-T and 150  $\mu\text{l}$  of the enzyme substrate *p*-nitrophenyl phosphate (1 mg/ml in diethanolamine substrate buffer; Sigma Chemical) was added to each well. Plates were protected from light during the enzyme substrate reaction, which was terminated after 15 min by adding 50  $\mu\text{l}$  of 1.5 M NaOH to each well. The optical density

(OD) of each well was determined using a plate reader equipped with a 405-nm wavelength filter (Bio-Rad model 3550), and the average OD for each set of duplicate wells was calculated. To minimize intra- and interassay variability, the average OD for each sample was expressed as a percentage of its plate-positive control OD for statistical analyses.

**Statistical analyses.** Each experimental variable was analyzed using a two (age) × two (capsule) × two (injection) × three (day) between-subjects analyses of variance (ANOVA). Any pairwise comparisons of mean differences were conducted using planned comparisons. Differences between groups were considered statistically significant at  $P < 0.05$ . Due to a high rate of mortality in aged animals, there were insufficient animals to analyze *day 15* data for this group.

**RESULTS**

**IgG response.** Mice immunized with KLH had increased anti-KLH IgG levels across days postimmunization ( $F_{2,54} = 41.99$ ;  $P < 0.05$ ) (Fig. 1). Animals treated with exogenous melatonin capsules did not differ from control mice receiving empty capsules in IgG levels ( $P > 0.05$ ). Both adult and aged mice exhibited comparable levels of anti-KLH IgG ( $P > 0.05$ ) (Fig. 1).

**Tissue mass and body temperature.** There were no significant differences in body mass between animals in

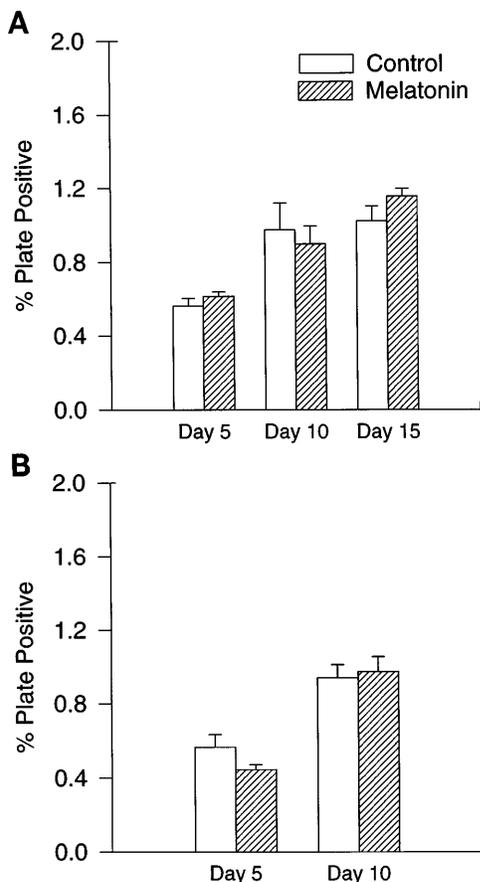


Fig. 1. Mean ( $\pm$ SE) serum anti-keyhole limpet hemocyanin (KLH) levels in adult (A) and aged (B) mice treated with melatonin or control mice from samples taken 5, 10, or 15 days postimmunization. Data are presented as percent plate positive, in which the mean of each sample was divided by the positive control run on the same microtiter plate.

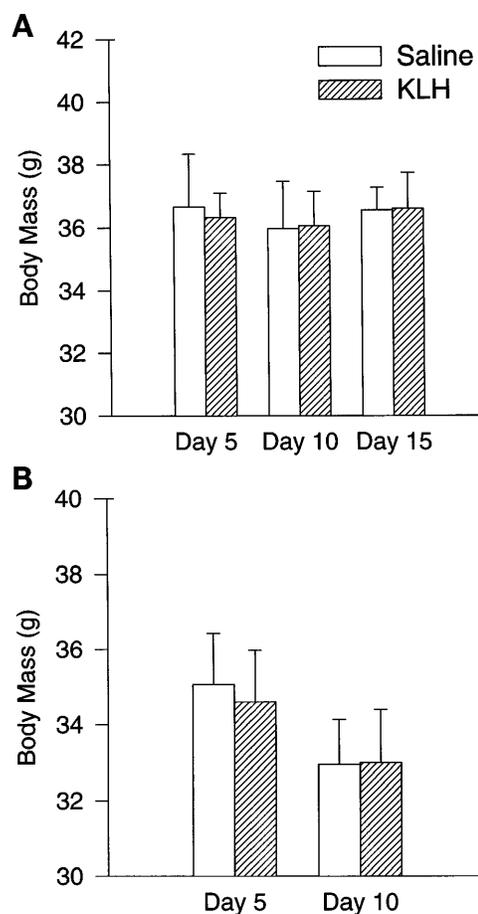


Fig. 2. Mean ( $\pm$ SE) body mass in adult (A) and aged (B) mice immunized with KLH or a saline control.

any experimental group ( $P > 0.05$ ) (Fig. 2). Adult mice had significantly larger paired testes mass ( $F_{1,124} = 97.94$ ;  $P < 0.05$ ) and BAT ( $F_{1,124} = 51.99$ ;  $P < 0.05$ ) (Table 1) but smaller splenic masses ( $F_{1,124} = 3.88$ ;  $P < 0.05$ ) compared with aged mice (Table 1). There were no differences in any tissue masses within either the adult or aged groups of animals ( $P > 0.05$  in all cases).

Colonic temperature did not differ between adult and aged mice ( $P > 0.05$ ) (Fig. 3). Mice injected with KLH had significantly higher colonic temperatures postim-

Table 1. Splenic mass and brown adipose tissue mass in adult or aged mice given Silastic implants containing melatonin or empty capsules and injected with KLH or saline

	Melatonin		Control	
	KLH	Saline	KLH	Saline
<i>Splenic mass</i>				
Adult	106 $\pm$ 12	144 $\pm$ 37	132 $\pm$ 29	116 $\pm$ 27
Aged	151 $\pm$ 36	166 $\pm$ 57	158 $\pm$ 41	146 $\pm$ 33
<i>Brown adipose tissue</i>				
Adult	148 $\pm$ 14	125 $\pm$ 17	143 $\pm$ 15	157 $\pm$ 14
Aged	67 $\pm$ 13	24 $\pm$ 10	71 $\pm$ 13	74 $\pm$ 12

Values are means  $\pm$  SE (in mg). KLH, keyhole limpet hemocyanin.

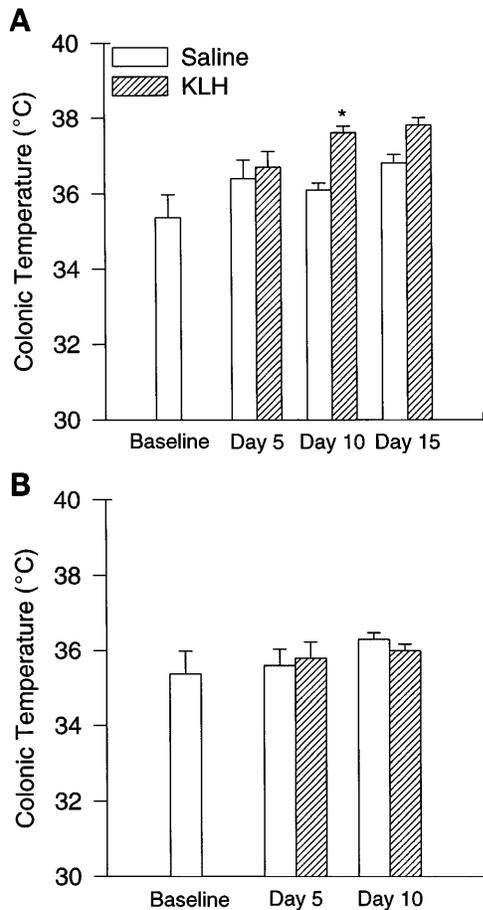


Fig. 3. Mean ( $\pm$ SE) colonic temperature in adult (A) and aged (B) mice immunized with KLH or a saline control, as assessed by a rectal thermoprobe. \*Significant differences between group means.

munization compared with saline-injected mice ( $F_{1,124} = 6.20$ ;  $P < 0.05$ ). Post hoc analysis revealed that this effect was due to significantly higher colonic temperatures in adult animals injected with KLH compared with saline-injected mice on *day 10* (Fig. 3). There were no other significant pairwise comparisons within adult animals. Colonic temperature did not differ in aged animals in any experimental group ( $P > 0.05$  in all cases) (Fig. 3).

**Oxygen consumption.** Mice injected with KLH displayed higher  $O_2$  consumption than mice injected with saline ( $F_{1,124} = 8.37$ ;  $P < 0.05$ ) (Fig. 4). Adult and aged mice did not differ in  $O_2$  consumption on any of the test days (e.g., *days 5, 10, and 15* postimmunization) ( $P > 0.05$  in all cases). Melatonin-treated animals had higher  $O_2$  consumption than mice receiving empty Silastic capsules ( $F_{1,124} = 6.69$ ;  $P < 0.05$ ). There was a significant interaction between type of capsule and injection ( $F_{1,124} = 3.62$ ;  $P < 0.05$ ). Adult mice receiving empty Silastic capsules and injected with KLH had higher  $O_2$  consumption on both *day 10* and *day 15* postimmunization compared with control mice injected with saline ( $P < 0.05$  in both cases). Adult KLH- and saline-injected mice did not differ in  $O_2$  consumption on *day 5* ( $P > 0.05$ ) (Fig. 4). Adult mice implanted with Silastic capsules containing melatonin and injected

with KLH showed no differences in  $O_2$  consumption compared with saline-injected mice on any of the three test days ( $P > 0.05$  in all cases). Aged mice receiving empty Silastic capsules and injected with KLH displayed higher  $O_2$  consumption on *day 10* postinjection ( $P < 0.05$ ). Aged KLH- and saline-injected mice did not differ in  $O_2$  consumption on *day 5* postinjection ( $P > 0.05$ ). Aged mice implanted with Silastic capsules containing melatonin and injected with KLH displayed no differences in  $O_2$  consumption compared with saline-injected mice on any of the three test days ( $P > 0.05$  in all cases) (Fig. 4).

## DISCUSSION

The primary finding of this study is that adult and aged mice immunized with KLH expended significantly more  $O_2$  than control mice injected with saline. Both adult and aged animals injected with KLH generated equally robust antibody responses and displayed significant increases in energy expenditure. Furthermore, melatonin treatment did not have significant effects on the generation of an antibody response, but did alter the metabolic response to the antigen. This was true in both adult and aged mice. These results suggest that mounting an immune response is energetically costly and that melatonin may mediate this energetic response. However, these results are contrary to previous suggestions that melatonin facilitates immune responses in mice.

Adult mice had higher colonic temperatures on *day 10* compared with *day 5* and *day 15* postimmunization; aged mice had no comparable increase in colonic temperatures. This result indicates that despite a significant increase in  $O_2$  consumption and therefore increased metabolic heat production, aged mice may not be able to raise body temperatures; failure to increase body temperature is likely due to increased heat loss in aged animals (e.g., Ref. 30). Taken together, these results demonstrate that mounting an immune response likely requires a significant energetic investment. KLH was chosen as an antigenic stimulus because it generates a quantifiable antibody response without making the animals ill. However, KLH is a relatively mild antigen that causes limited activation of the immune system (11); it is likely that the energetic costs assessed in the present study would be greatly increased with the use of more ecologically relevant antigenic challenges (i.e., bacteria, parasites). Analogously, adult and aged mice respond similarly to moderate cold stress, but aged animals do not cope as well as younger animals with severe cold stress (36).

Nevertheless, melatonin-treated mice expended more energy (e.g., increased  $O_2$  consumption) compared with untreated control mice. However, melatonin-treated animals injected with KLH failed to display increased  $O_2$  consumption relative to melatonin-treated control mice injected with saline. Thus melatonin appears to suppress the metabolic, but not the immune, response to KLH in mice. In aged mice, melatonin reduced BAT depots; immunization with KLH in aged mice increases BAT mass to pre-melatonin treatment levels. These

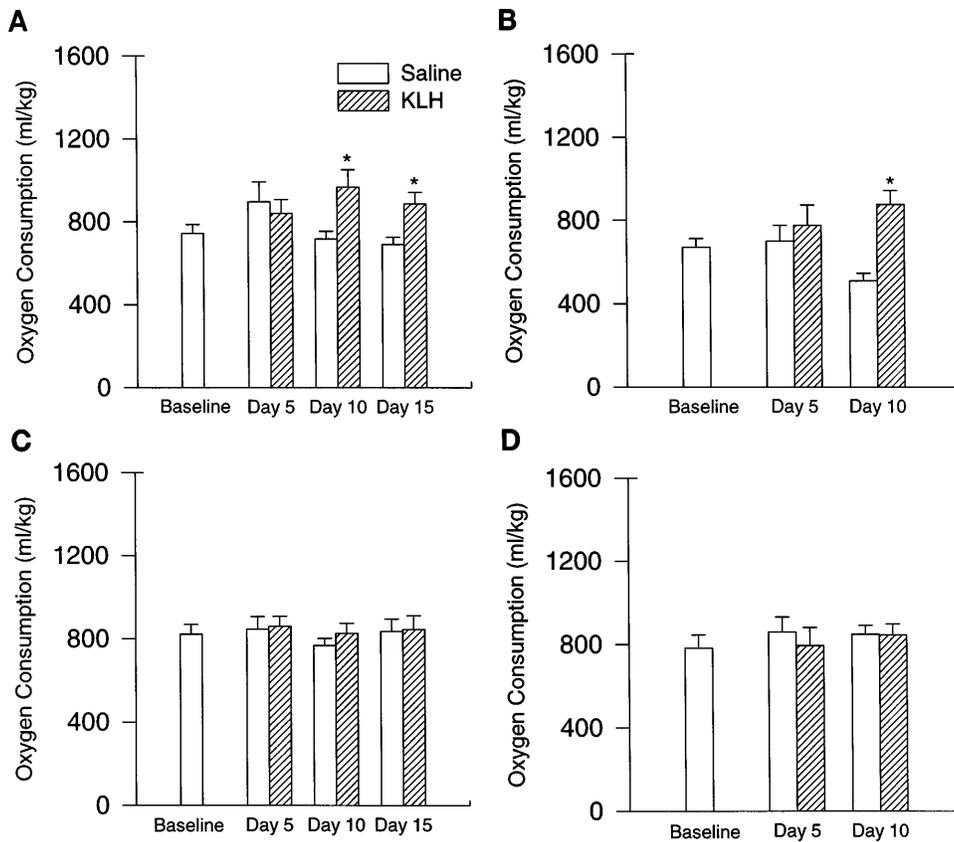


Fig. 4. Mean ( $\pm$ SE)  $O_2$  consumption in adult mice receiving empty capsules (A), aged mice receiving empty capsules (B), adult mice receiving melatonin capsules (C), or aged mice receiving melatonin capsules (D). \*Significant differences between group means.

results are consistent with the hypothesis that melatonin plays a role in coordinating reproductive, immunologic, and energetic processes rather than enhancing immune function directly (1, 8, 20, 28).

Mounting an immune response requires using resources that could otherwise be allocated to other physiological processes (e.g., thermoregulation, reproduction, immune function) (28). Melatonin may improve the efficiency of metabolic fuel use in response to an antigenic challenge, reducing the energetic cost associated with mounting an immune response. For example, exogenous melatonin improves thermogenic function in deer mice (4). Maintaining deer mice on short day lengths (which prolongs the duration of nightly melatonin secretion) also buffers against 2-deoxy-D-glucose-induced metabolic stress and increases immune function (8). Presumably, short-day animals experience improved metabolic function as a result of increased melatonin secretion.

Despite previous research demonstrating increased immune function in mice treated with melatonin (9, 15), melatonin had no effect on antibody production in the present study. Also, previous research has demonstrated that inbred strains of mice (including C57BL/6) have a genetic defect and are unable to synthesize melatonin (12, 14). Mice are ideal for immune studies, and it was necessary to establish that metabolic rate increased in response to antigen stimulation in a traditional animal model. Mice, like humans, are opportunistic and omnivorous and both species are commensal worldwide. However, because house mice are gener-

ally unresponsive to melatonin (32), they are not typically used as animal models for studies of seasonality. For example, administration of melatonin via subcutaneous Silastic capsules fails to reduce testicular mass in male mice; melatonin-sensitive species [i.e., golden hamsters (*Mesocricetus auratus*) and grasshopper mice (*Onychomys leucogaster*)] undergo significant testicular regression (32). Melatonin-responsive deer mice (*Peromyscus maniculatus*) treated with exogenous melatonin undergo reproductive regression and display enhanced immune function relative to untreated mice (9); deer mice that fail to respond to melatonin (i.e., melatonin insensitive) do not undergo reproductive regression and do not display enhanced immune function when given exogenous melatonin treatment (9). Thus reproductive responsiveness to melatonin may be required for the immunoenhancing effects of this indoleamine. The use of a melatonin-responsive species (e.g., deer mice, prairie voles, Siberian hamsters) will provide a better model than house mice for studying the effects of melatonin on immune function.

The notion that immune function is optimized based on available energy and competing energetic requirements is consistent with our results that suggest that immune function should be reduced when energy demands are high (i.e., Ref. 10). On the basis of our results, immune function should be generally compromised during winter, pregnancy, or lactation, particularly if energy availability is low. For example,  $O_2$  consumption during thermoregulation in small mammals increases  $\sim 30\%$  for every  $10^\circ\text{C}$  reduction in

ambient temperature (17) while the daily energetic cost of lactation is ~40% at peak levels (35). Our results also suggest that melatonin might maintain immune function during the energetic bottleneck associated with high winter thermogenic needs when food availability is often scarce. This hypothesis requires additional direct tests in the future with appropriate animal models of seasonal breeding. The adaptive functional perspective that immune function is optimized may also require a reassessment of the role of energetic "stress" on immune function; labeling a response such as immunocompromise when energy is low as a "stress response" likely obscures the energy-savings adaptive function of reduced immunity (see Ref. 27).

Aged mice had smaller testes and significant reductions in BAT mass. However, aged animals did not differ in total body mass compared with adult mice; thus specific tissue differences are not likely due to overall changes in body mass. These results confirm and extend previous findings of reductions in reproductive behavior, physiology, and morphology in aged house mice (6). Taken together, the present findings suggest that mounting an immune response requires significant energy. Both adult and aged mice demonstrate increased metabolic response to KLH. However, aged animals are unable to generate sufficient heat in response to an antigenic challenge compared with adult mice. Melatonin reduces metabolic rate, but not immune response to KLH. Thus melatonin appears to play a role in coordinating reproductive, immunologic, and energetic processes rather than enhancing immune function directly when antigenic stimulation is mild.

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